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Transient Expression of the *Autographa californica* Nuclear Polyhedrosis Virus Immediate-Early Gene, IE-N, Is Regulated by Three Viral Elements

DAVID D. CARSON,[1] MAX D. SUMMERS,[1,2,3] AND LINDA A. GUARINO[2,3]*

Departments of Biochemistry and Biophysics[1] and Entomology[2] and the Center for Advanced Invertebrate Molecular Science,[3] Texas A&M University, College Station, Texas 77843

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Autographa californica **nuclear polyhedrosis virus (Ac*M*NPV) is a double-stranded DNA virus that expresses several immediate-early genes under the control of different promoters. The expression of one of these transcription units, IE-N, is shown here, by a transient expression assay, to be regulated by both *cis*- and *trans*-acting viral elements. The steady-state levels of IE-N mRNA were very abundant soon after infection but were nearly undetectable during the late phase of the viral life cycle. Analysis of the transient expression of a reporter construct driven by the IE-N promoter (IE-NCAT) was conducted to define viral elements which regulate IE-N gene expression. Viral enhancer *hr*1 and two immediate-early genes, IE-1 and IE-N, were shown to affect relative levels of reporter enzyme activity produced by IE-NCAT. The *hr*1 enhancer stimulated the expression of IE-NCAT, independent of orientation and position relative to the promoter and in the absence of any *trans*-acting viral factors. Regulation of IE-NCAT expression by the IE-1 and IE-N genes required less than 290 bp of promoter sequences upstream of the site of transcription initiation and was not dependent upon the *hr*1 enhancer. Coexpression of the IE-N gene had an autostimulatory effect upon IE-NCAT activity, whereas coexpression of the IE-1 gene reduced levels of reporter activity. The levels of reporter activity measured upon coexpression of either immediate-early gene with IE-NCAT linked to the *hr*1 enhancer appear to be the combined result of both *cis*- and *trans*-regulatory elements influencing expression from IE-NCAT. These results suggest that IE-N gene expression in baculovirus infection may be influenced by the concerted activity of three Ac*M*NPV regulatory elements.**

In insect cells infected with *Autographa californica* nuclear polyhedrosis virus (Ac*M*NPV), viral genes are expressed in a defined temporal pattern. By the use of transient assays, three viral elements which may regulate the expression of delayed-early genes in infected cells (2, 9, 10) have been defined. The immediate-early gene IE-1 was functionally mapped by its ability to *trans* activate a delayed-early reporter construct in transient expression assays (9). Steady-state levels of IE-1 mRNA are detectable very soon after infection and persist until at least 24 h postinfection (4, 11). Five regions of homologous DNA (*hr*s) were shown to function as enhancers of delayed-early gene expression (10). The *hr* enhancers were first noticed as repetitive DNA sequences which cross-hybridized (5). Each of the *hr*s was subsequently cloned and sequenced (8). Stimulation of delayed-early gene expression by the five viral enhancers requires the activity of IE-1 (9).

The *trans* activation of delayed-early gene expression by IE-1 is augmented by a second immediate-early gene, IE-N (2). IE-N activity was transiently expressed by the *Pst*I N fragment of Ac*M*NPV. Expression of the IE-N gene product was directed by 280 bp of the *Pst*I N fragment upstream of the 5′ terminus of the IE-N transcript. The IE-N gene was defined as an immediate-early gene by its temporal expression during the viral life cycle and by transient expression of IE-N in the absence of other viral genes. However, steady-state levels of IE-N mRNA isolated from *Spodoptera frugiperda* cells soon after infection with Ac*M*NPV were much higher than levels of mRNA from cells transiently expressing pPstI-N (2). This difference in levels of mRNA may be due to the fact that infection is a more efficient process than transfection. However, elements present in viral DNA that are not in the plasmid may also contribute to the higher levels of expression in infection.

The objectives of this study were to determine the relative levels of IE-N expression during Ac*M*NPV infection and to define viral regulatory elements which may influence IE-N gene expression. Analysis of RNA isolated from infected cells at various times postinfection indicated that IE-N mRNA levels steadily decreased during early stages of viral infection until they were nearly undetectable during the late phase. These observations suggested that IE-N expression was regulated by *cis*- or *trans*-acting viral elements. Transient expression assays defined three elements of the Ac*M*NPV genome which regulate IE-N gene expression: *hr*1, IE-1, and IE-N.

Addition of the *hr*1 enhancer to the basal-level IE-NCAT reporter construct stimulated chloramphenicol acetyltransferase (CAT) activity 10- to 20-fold. This *cis* activation was independent of orientation and position relative to the IE-NCAT promoter. Addition of IE-1 to the IE-NCAT constructs decreased the overall expression of IE-N, independent of the presence of the complete *hr*1 enhancer. However, deletion mutants of the *hr*1-linked IE-NCAT constructs which lack the *cis*-stimulatory activity of the enhancer are *trans* activated by the IE-1 gene. The IE-N gene product appears to stimulate its own production in an enhancer-independent fashion. Thus, at least three viral elements influence the transient expression of IE-N in insect cell culture.

* Corresponding author.

MATERIALS AND METHODS

Cell culture. *S. frugiperda* cells (Sf9) were cultured and transfected with cloned recombinant DNAs as described by Guarino and Summers (9) and Summers and Smith (16). *S. frugiperda* cells were infected with Ac*M*NPV E2 at a multiplicity of infection of 20. The virus was adsorbed for 1 h at 27°C, after which the inoculum was removed and the cells were incubated at 27°C until harvest. Zero hours postinfection (hpi) is defined as the end of the adsorption period.

RNA analysis. Total cellular RNA was isolated from *S. frugiperda* cells by the method of Chirgwin et al. (3) as described previously (9). S1 nuclease mapping of Ac*M*NPV RNA was performed by the method of Berk and Sharp (1) and Favaloro et al. (6), by utilizing 5′- and 3′-end-labeled DNA probes generated by the methods described by Maniatis et al. (14). The DNA probes were hybridized to RNA at 45°C for 16 h following denaturation at 80°C for 15 min. Nonhybridized molecules were digested with 166 U of S1 nuclease per ml at 37°C for 30 min. Protected fragments were separated by 4% (7 M urea) polyacrylamide gel electrophoresis. Sizes of fragments were determined by using 3′-end-labeled FX-174 *Hae*III fragments as molecular standards.

Plasmid constructions. The plasmids pPstI-N, pPstI-NCAT, pIE-1, and p*hr*1 have been previously described (2, 8, 9). pHindIII-F is the 8.7-kb fragment of the *Hind*III-cleaved Ac*M*NPV E2 DNA cloned into the *Hind*III site of pUC8. A *Bgl*II site was inserted into the polycloning site of the Blue-Script SK (+) cloning vector (Stratagene Cloning Systems, La Jolla, Calif.) by digestion with *Bam*HI, repairing the 5′ protruding ends by treatment with DNA polymerase I large fragment (Klenow) in the presence of all four nucleotides, and ligating that DNA to *Bgl*II linkers (5′-CAGATCTG-3′) obtained from New England BioLabs, Beverly, Mass. This modified cloning vector was designated pBSSK-Bgl and contains a unique *Bgl*II site as well as the regenerated *Bam*HI site.

pHindIII-FCAT was constructed by ligating the 3.5-kb *Pst*I fragment of pPstI-NCAT and the 8.8-kb fragment of pHindIII-F digested with *Pst*I. This regenerates the 6.1 kb of sequences upstream of the IE-NCAT chimeric gene encoded by pPstI-NCAT.

Deletion mutants of pHindIII-FCAT were constructed with the use of convenient restriction sites in the *Hin*dIII-F fragment. pFCATΔXhoI, pFCATΔBstEII, and pFCATΔ BglII were made by digesting pHindIII-FCAT with *Hin*dIII and then with *Xho*I, *Bst*EII, and *Bgl*II, respectively, and treating the resulting 5′ protruding ends with DNA polymerase I large fragment (Klenow) in the presence of all four nucleotides; the large DNA fragments were isolated from a low-melting-point agarose gel and recircularized with T4 DNA ligase. pFCATΔAsuII and pFCATΔClaI were constructed by subcloning the 5.8-kb *Bam*HI-*Asu*II fragment and the 4.4-kb *Bam*HI-*Cla*I fragment of pHindIII-FCAT into the *Bam*HI and *Acc*I sites of pUC18 (17). pFCATΔEcoRV was constructed by digesting pHindIII-FCAT to completion with *Bam*HI and then partially digesting it with *Eco*RV. The resulting 4.7-kb fragment was purified from low-melting-point agarose and cloned into pUC18 previously digested with *Bam*HI and *Hin*cII. pFCATΔEcoRI was constructed by partially digesting pHindIII-FCAT with *Eco*RI restriction enzyme, purifying the resulting 4.6-kb fragment from low-melting-point agarose gel, and subcloning it into pUC18. pFCATΔScaI was constructed by inserting the 0.776-kb *Bam*HI fragment containing the CAT open reading frame from pCAT (13) into the unique *Bgl*II site of pBSN(+) (2) and screening by restriction site analysis for proper orientation.

The 3.6-kb *Bgl*II-*Xho*I fragment of pHindIII-F (containing *hr*1) was subcloned into pBSSK-Bgl. Deletion mutants of *hr*1 were constructed by digesting this plasmid with excess *Eco*RI restriction endonuclease, gel purifying the resulting large DNA fragment, and ligating the DNA fragment. Three deletion mutants were selected by restriction fragment analysis, and the *Bgl*II-*Xho*I fragment was cloned into pHindIII-FCAT, replacing the wild-type *hr*1 with the mutants deleted in *hr*1. The enhancer regions of these deletion mutants, designated pFCATDA, pFCATDB, and pFCATDC, were sequenced with double-stranded templates and Sequenase 2.0 (United States Biochemical, Cleveland, Ohio). Sequencing primers specific to the flanking sequences of *hr*1,

*hr*1-left (5′-GACACAGCAACATACAATTCTTG-3′)

*hr*1-right (5′-GTTGTGCCAAGCGCGGGAAAC-3′)

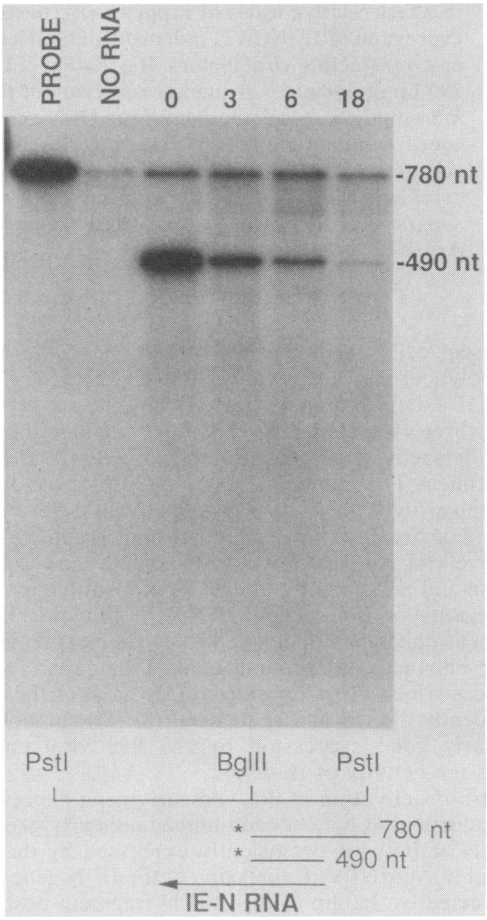

FIG. 1. S1 nuclease analysis of IE-N mRNA at various times during Ac*M*NPV infection. Total RNA was extracted from *S. frugiperda* cells 0, 3, 6, or 18 hpi and hybridized to the 780-nt *Bgl*III-*Pst*I DNA fragment labeled with ^{32}P at the *Bgl*II 5′ terminus. IE-N mRNA protects a 490-nt portion of the probe upon degradation by S1 nuclease. Undegraded probe and probe incubated with S1 nuclease in the absence of RNA were used in the first two lanes, respectively. The positions of 780-nt probe and the 490-nt protected fragment are indicated on the right. A diagram of the probe, protected fragment, and mRNA is shown below the gel. *, Position of the ^{32}P label.

were synthesized with a 391 DNA synthesizer (Applied Biosystems, Foster City, Calif.). The 0.874-kb *Cla*I fragment of pHindIII-FCAT was excised and inserted in the opposite orientation to create pHindIII-FCAThr1FLIP. pPstI-NCAThr1 was constructed by cloning the 3.5-kb *Pst*I fragment of pPstI-NCAT into p*hr*1.

CAT assays. CAT assays were performed as described by Gorman et al. (7) except for the modifications detailed by Guarino and Summers (9). Typically, cell extracts were diluted so that the maximum amount of chloramphenicol acetylated in any sample was not more than 60%. The chloramphenicol was separated by ascending thin-layer chromatography and exposed to Kodak XRP film at room temperature overnight. The unreacted substrate chloramphenicol and the acetylated chloramphenicol were quantitated by liquid scintillation counting. The average level of CAT expression in cells transfected with IE-NCAT was 6.5 pmol/min/10^6 cells.

RESULTS

IE-N is highly expressed early, but not late, during Ac*M* NPV infection. Total cellular RNA isolated at various times during infection was analyzed by S1 nuclease protection assays. The 780-nucleotide (nt) *Pst*I-*Bgl*II 5′-end-labeled DNA probe, which protects a 490-nt fragment after hybridization with IE-N mRNA, was used to detect steady-state levels of IE-N message in infected *S. frugiperda* cells (Fig. 1). IE-N message was abundant early in infection (from 0 to 3 hpi), was less abundant at 6 h, and was nearly undetectable late in infection (18 hpi). To define *cis*- and *trans*-acting viral elements which might influence the levels of expression of IE-N during viral infection, transient expression assays were performed with deletion mutants of pHindIII-FCAT.

Viral sequences upstream of *Pst*I-N stimulated transient expression of IE-N. pHindIII-FCAT encodes the IE-NCAT fusion gene along with 6.1 kb of upstream sequences (Fig. 2). Of note within these sequences is the 874-bp *Cla*I fragment containing *hr*1, 2.0 kb upstream of the IE-N transcription unit. With the use of convenient restriction enzyme sites, portions of the upstream region were deleted. Five of these deletion constructs contained *hr*1 (or a portion of it), and five did not contain *hr*1 sequences. When these constructs were transiently expressed in *S. frugiperda* cells, an increase in CAT activity correlated with the presence of upstream sequences (Fig. 3). The addition of 6.1 kb of Ac*M*NPV DNA sequences provided by pHindIII-FCAT stimulated IE-NCAT expression greater than 10-fold over that observed for pPstI-NCAT. All deletion constructs retaining at least 2.7 kb of upstream sequences, and therefore the entire *hr*1 region, expressed increased levels of CAT activity relative to that of pPstI-NCAT. Those constructs lacking the *hr*1 region exhibited activity equal to or less than that of pPstI-NCAT. pHindIII-FCATDEcoRV, which contains two of the five enhancer repeats of *hr*1, exhibited a level of activity (twofold stimulation) intermediate between those of clones containing the enhancer and those lacking the enhancer. No CAT activity was detected with the smallest clone (pHindIII-FCATDScaI), which contained only 0.045 kb of upstream sequences.

The viral enhancer *hr*1 stimulated IE-NCAT activity in *cis*. The *hr*1 enhancer had previously been shown to *cis* activate a delayed-early gene (10). To determine whether this region

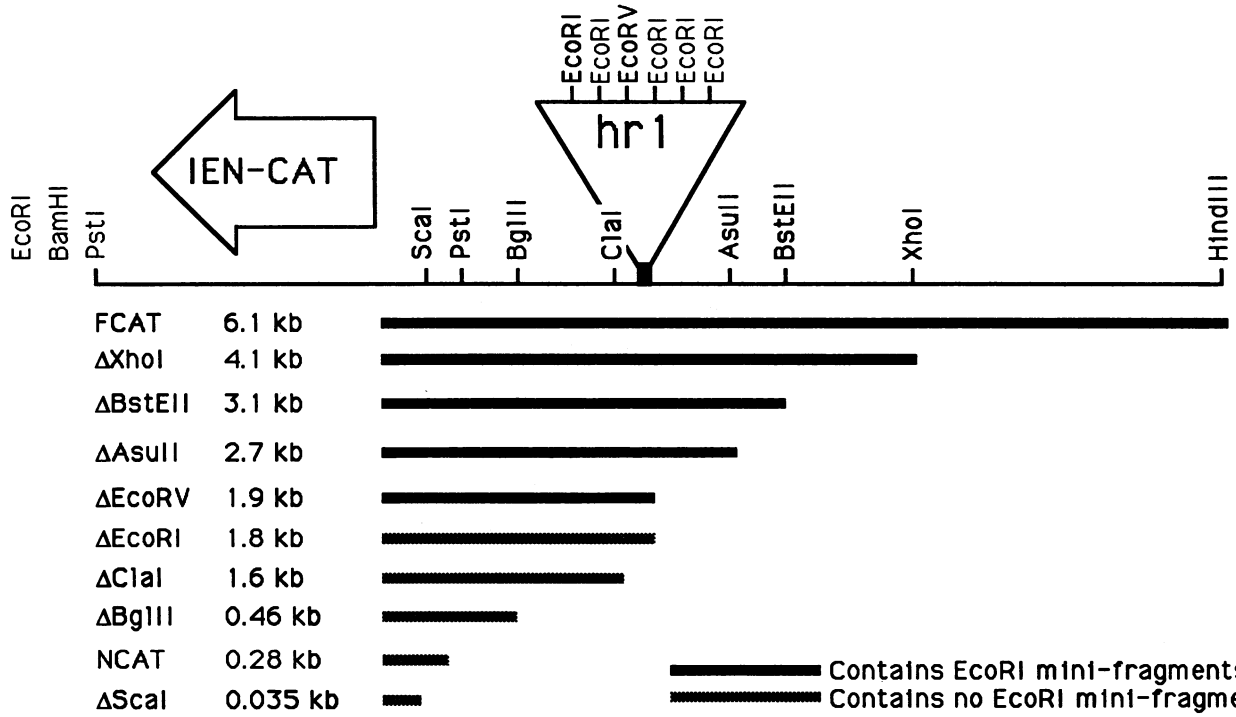

FIG. 2. Deletion clones of upstream DNA sequences in pHindIII-FCAT. The linear map of pHindIII-FCAT indicates salient features of the constructs, including the location and orientation of IE-NCAT transcription and the viral enhancer *hr*1. Restriction sites in boldface indicate those sites delineating the 5′ boundary of deletion mutants. The restriction sites of *hr*1 are indicated on the expanded portion above the map. The abbreviated name of the deleted construct and the amount of DNA sequence added 5′ to the transcription start site are given to the left of each bar. Symbols: ▬, clones containing all or part of *hr*1; ▬, constructs which contain none of the viral enhancer.

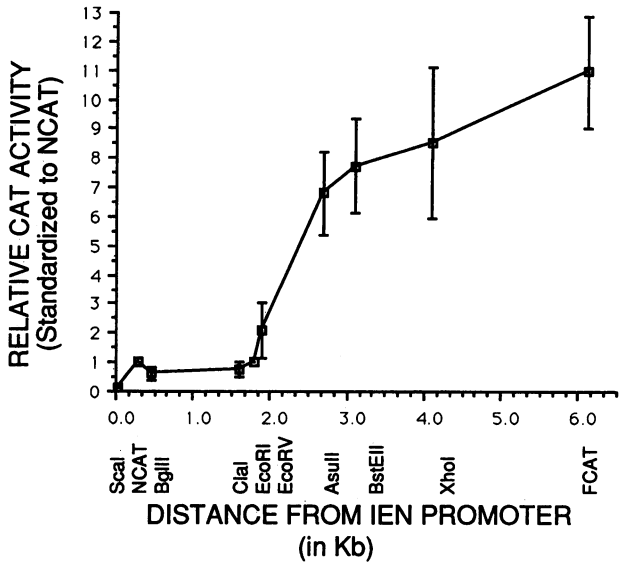

FIG. 3. Stimulation of IE-NCAT expression by upstream DNA sequences. A total of 10^6 *S. frugiperda* cells were transfected with equimolar amounts of pHindIII-FCAT or deletion mutants and assayed for CAT activity after 24 h. The values shown are the means of four repetitions, and error bars indicate plus and minus standard error. The amounts (in kilobases) of upstream sequences contained in the pHindIII-FCAT deletion mutants are indicated on the horizontal axis. The relative units of CAT activity, standardized to the activity expressed by pPstI-NCAT, are shown on the vertical axis. The pHindIII-FCAT deletion mutants are indicated below the graph.

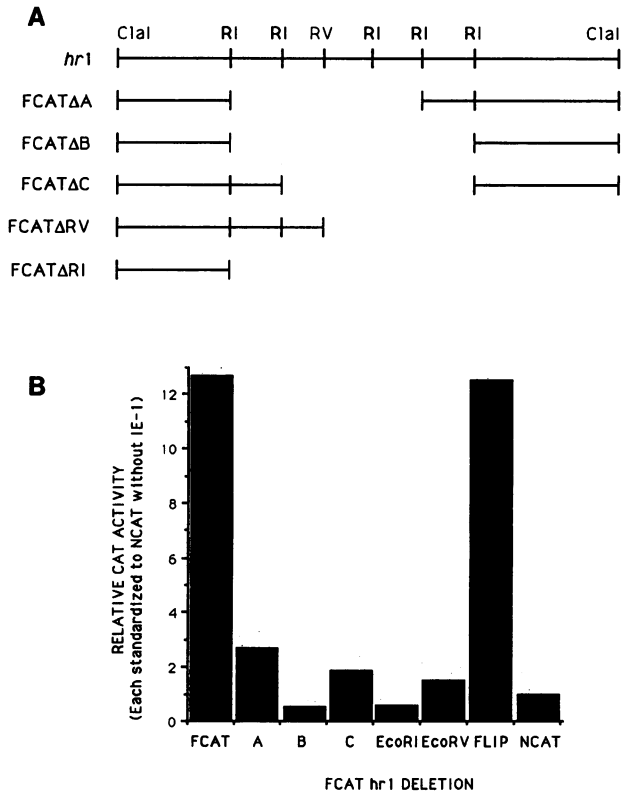

FIG. 4. (A) Restriction fragment map of pHindIII-FCAT *hr*1 deletion mutants. The map of the *hr*1 enhancer is shown with the *Cla*I, *Eco*RI (RI), and *Eco*RV (RV) sites indicated by vertical lines. Represented below are portions of the 874-bp *Cla*I fragment replacing the native *hr*1 sequences in the designated pHindIII-FCAT mutant. (B) Decrease in transient expression from pHindIII-FCAT by deletions in *hr*1. A total of 10^6 *S. frugiperda* cells were transfected with equimolar amounts of the clones indicated below the bars. All CAT activities were standardized to that of pPstI-NCAT.

was responsible for the observed stimulation of IE-N expression, deletion mutants with precisely removed internal sequences and retained sequences further upstream of *hr*1 were made. Figure 4A graphically depicts three constructs which lack portions of the *hr*1 sequences but retain all other sequences in the *Hin*dIII F fragment. The nucleotide sequences of pHindIII-FCATΔA, pHindIII-FCATΔB, and pHindIII-FCATΔC were determined and compared with the previously published sequence of *hr*1 (9). Two constructs (pHindIII-FCATΔA and pHindIII-FCATΔC) each retained one *Eco*RI minifragment, whereas one construct (pHindIII-FCATΔB) was depleted of all internal *Eco*RI minifragments. Shown below the internal enhancer deletions are pHindIII-FCATΔEcoRV and pHindIII-FCATΔEcoRI, which have *hr*1 deleted but contain no sequences upstream of *hr*1.

pHindIII-FCAT *hr*1 deletion constructs ΔA, ΔB, and ΔC were transfected separately into *S. frugiperda* cells and assayed for activity (Fig. 4B). The deletion of any portion of *hr*1 resulted in an at least 10-fold reduction of activity compared with the CAT activity expressed by pHindIII-FCAT. The levels of expression of the *hr*1 deletion constructs were comparable to those of pHindIII-FCAT-DEcoRI, pHindIII-FCATDEcoRV, and pPstI-NCAT, which lack part or all of the enhancing sequences of *hr*1. When the orientation of *hr*1 was reserved (pHindIII-FCAThr1FLIP), the level of activity was equal to that of pHindIII-FCAT.

To determine whether the sequences added to the IE-NCAT transcription unit were acting in *cis* or *trans*, the 6.1 kb of upstream sequences was cloned into pUC18 (pPstI-HindIII) and cotransfected with pPstI-NCAT, and the result was compared with those for pPstI-NCAT or pHindIII-FCAT transfected alone (Fig. 5). When IE-NCAT was transiently expressed by pPstI-NCAT in the presence of pPstI-HindIII, i.e., the upstream sequences and IE-NCAT transcription unit were on different molecules, CAT activity was not stimulated to a level higher than that for the activity of pPstI-NCAT alone. When upstream sequences were present on the same molecule as the transcription unit (pHindIII-FCAT), activity was stimulated 12-fold. Finally, when the 874-nt *Cla*I fragment containing *hr*1 was cloned directly upstream of the IE-N promoter in pPst-NCAT, levels of CAT expression were increased to match those of pHindIII-FCAT. These data indicated that the *hr*1 enhancer sequences upstream of *Pst*I-N in the *Hin*dIII F fragment were both necessary and sufficient to stimulate IE-N expression in *cis*.

IE-1 interacted with *hr*1 sequences to affect IE-N expression in *trans*. The *hr*1 enhancer linked to a delayed-early gene promoter requires the presence of IE-1 for its activity. Therefore, the effect of adding IE-1 to IE-NCAT constructs with and without the *hr*1 enhancer was tested. *S. frugiperda* cells were transfected with pHindIII-FCAT deletion constructs in the presence or absence of IE-1. Figure 6 shows the result of adding pIE-1 to insect cells expressing pHindIII-FCAT deletions. In the presence of IE-1, the constructs containing the entire *hr*1 enhancer expressed approximately twofold less CAT activity than in the absence of IE-1. Those deletion constructs lacking the *hr*1 enhancer also showed

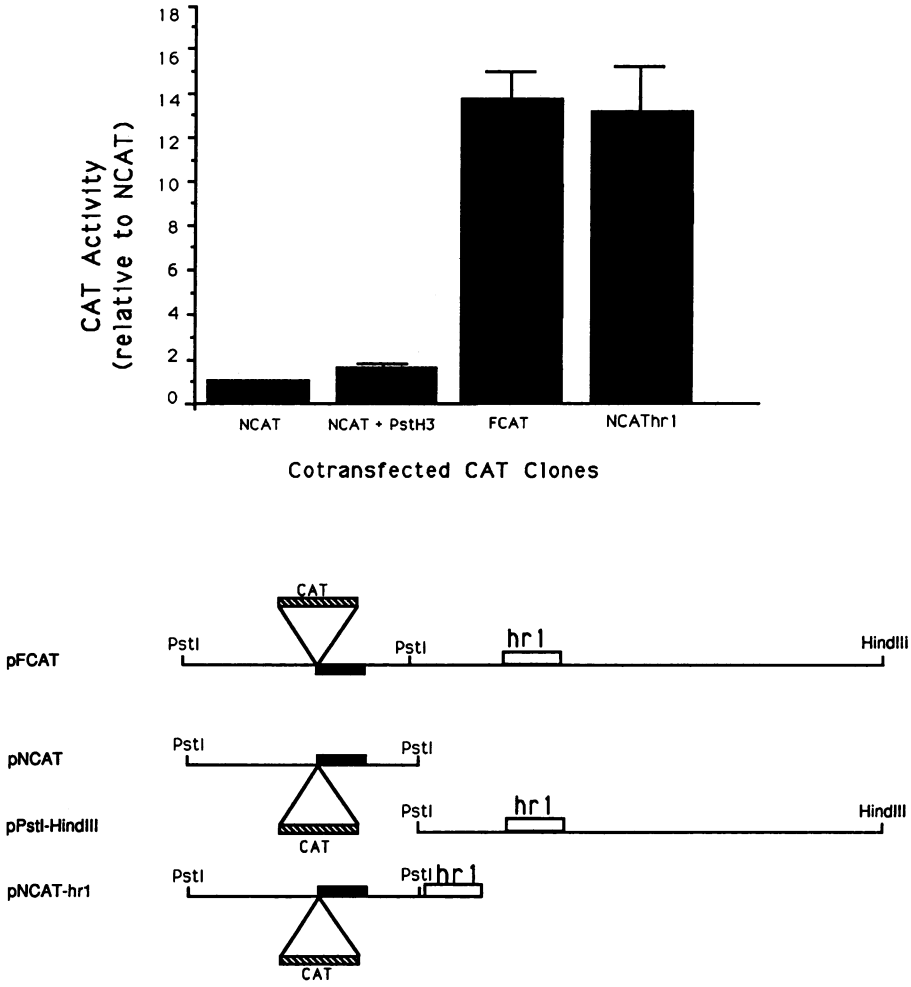

FIG. 5. Stimulation of IE-NCAT expression only in *cis* by the *hr*1 enhancer. A total of 10^6 *S. frugiperda* cells either were transfected with pPstI-NCAT, pHindIII-FCAT, or pPstI-NCAThr1 alone or were cotransfected with pPstI-HindIII and pPstI-NCAT. The vertical axis shows CAT activity relative to that of pPstI-NCAT alone. Values shown are the means of three replicates plus and minus standard error. Shown below the bar graph are diagrams of the transfected plasmids. The *hr*1 enhancer and the IE-NCAT open reading frame are indicated.

lower levels of CAT activity upon addition of pIE-1 to the cells. Interestingly, pHindIII-FCATΔEcoRV, which possesses part of the *hr*1 enhancer (two of five *Eco*RI minifragments in the enhancer), showed four- to fivefold stimulation compared with the construct transfected alone. This suggested that some sequence within or adjacent to *hr*1 obviated the *hr*1-mediated stimulation by IE-1 and allowed inhibition of IE-N gene expression.

The IE-N gene product autoregulated its own production, independent of the presence of the *hr*1 enhancer. To determine whether the IE-N protein had any effect upon expression from its own promoter, pPstI-N, which can express the intact IE-N protein, was cotransfected with either pPstI-NCAT or pHindIII-FCAT. Coexpression of IE-N with IE-NCAT stimulated levels of CAT activity 2.5-fold (Fig. 7A). This stimulation was not observed when the IE-NCAT construct was cotransfected with pPstI-N (Bgl). This plasmid lacks detectable IE-N activity (2), presumably because of a frameshift mutation introduced at the *Bgl*II site in the IE-N gene. Linking the *hr*1 enhancer to IE-NCAT (pHindIII-FCAT) did not change the level of activation of CAT activity induced by coexpression of intact IE-N (Fig. 7B). The level of activity expressed by pHindIII-FCAT in the presence of IE-N was 2.5- to 3.5-fold greater than that expressed by pHindIII-FCAT. The overall stimulation of IE-NCAT expression in the presence of both the enhancer and the IE-N gene product was 30-fold greater than the stimulation of IE-NCAT in the absence of both viral elements.

DISCUSSION

Expression of the IE-N gene product from the cloned *Pst*I N fragment of the Ac*M*NPV genome was previously shown to occur in uninfected cells and, therefore, in the absence of any viral gene expression (2). However, the levels of IE-N message varied during infection. IE-N message was maximally expressed very early in infection. The steady-state levels of mRNA decreased throughout the early phase of infection until levels of expression were nearly undetectable during the late phase (18 hpi). This decrease in IE-N mRNA levels during the viral life cycle contrasts with the pattern of expression observed for IE-1. IE-1 mRNA levels remain

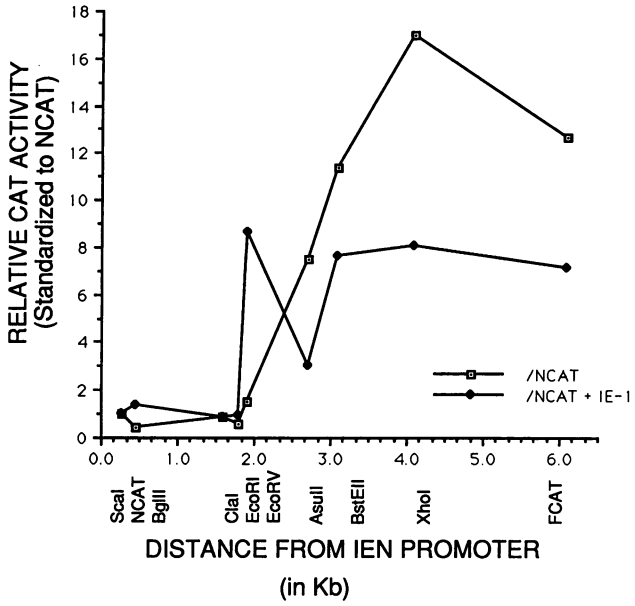

FIG. 6. Activities of pHindIII-FCAT deletion mutants in the presence or absence of IE-1. A total of 10^6 *S. frugiperda* cells were transfected separately with equimolar amounts of each pHindIII-FCAT deletion clone. CAT activities were standardized to that of NCAT. A duplicate set of transfections were cotransfected with 1.0 μg of pIE-1. CAT activities for these assays were standardized to that of NCAT cotransfected with pIE-1. Relevant enzyme sites of pHindIII-FCAT are indicated below the graph.

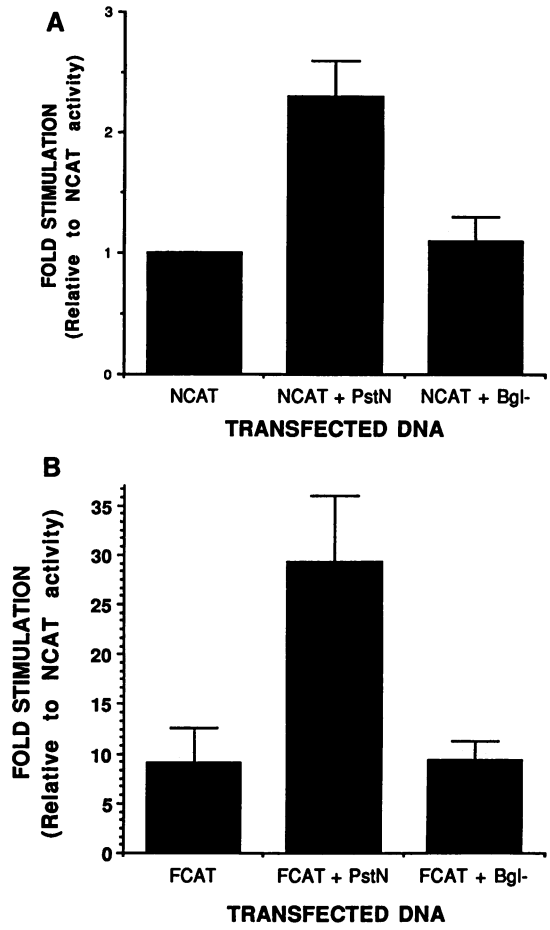

FIG. 7. Stimulation of IE-NCAT expression by the IE-N gene product. Stimulation of CAT activity expressed by pPstI-NCAT cotransfected with pPstI-N or pPstI-N (Bgl) (A) and by pHindIII-FCAT cotransfected with pPstI-N or pPstI-N (Bgl) (B) relative to that of pPstI-NCAT alone is shown. The values reported are the means of four replicates, with the standard error shown by bars.

relatively constant throughout baculovirus infection (4, 11, 12).

To begin to understand viral elements that regulate expression of IE-N, transient assay experiments with an IE-N reporter construct were conducted. The regulation of IE-N expression in uninfected insect cells was influenced by both *cis*- and *trans*-acting viral elements. Three elements of the Ac*M*NPV genome influence IE-N gene expression in transient assays: the *hr*1 enhancer and the IE-1 and IE-N regulatory genes.

The *hr*1 enhancer has previously been shown to activate transient expression from the delayed-early 39,000-molecular-weight promoter (39K promoter) 1,000-fold in the presence of IE-1 (8). The CAT activities expressed by *S. frugiperda* cells cotransfected with p39CAT linked to a homologous region and IE-1 were 200 to 1,900 times greater than that of the original p39CAT construct cotransfected with IE-1 (8). Each of these homologous regions was orientation independent in its ability to enhance expression of 39CAT. The *hr* enhancers were inactive unless they were cotransfected with IE-1.

The nucleotide sequences of all five homologous regions were determined (8). Each of the homologous regions was found to contain multiple copies of the highly conserved palindrome. This palindrome consists of 26 bp about a central *Eco*RI recognition site with the consensus sequence TTTACaAGTAGAATTCTACTcGTAAA (where the *Eco*RI restriction site is underlined and the imperfect elements of the palindrome are lowercase). Analysis of *Bal* 31 deletion mutants of *hr*5 showed that the presence of only one of these conserved palindromes was sufficient for enhanced gene expression (8) induced by IE-1.

In this study, we demonstrate that *hr*1 stimulated expression from the immediate-early IE-N promoter 12-fold in the absence of IE-1. The *hr*1 DNA sequence was also shown to act only in *cis* and not in *trans* and to be both orientation and position independent in its enhancing activity. In these transient assays of *hr*1 linked to IE-NCAT, enhancer function was not dependent upon IE-1. Nissen and Friesen (15) reported similar activation of the early 35K gene *cis* linked to *hr*5 and in the absence of IE-1. However, the 35K gene construct was transiently expressed only in the presence of either the *hr*5 enhancer or the IE-1 gene product but was not expressed at levels above background in the absence of both stimulating elements.

When IE-1 was added to IE-NCAT, levels of expression were decreased. This effect was seen in both the presence and the absence of the enhancer. Taken together, these data suggest that IE-1 can act independent of the enhancer to negatively regulate IE-N expression and that IE-1 and host cellular factors regulate *hr*1-mediated enhancement in a complex fashion.

The autoregulation of IE-N expression by its own gene product, on the other hand, appears to be mediated by sequences within the 280-bp basal-level promoter. The stimulation of expression was the same in the presence or

absence of the viral enhancer. The 30-fold increase of expression of the IE-NCAT construct linked to the enhancer in the presence of IE-N compared with that of the IE-NCAT construct alone represents the combination of the enhancer-mediated stimulation (10- to 20-fold) and the IE-N-induced stimulation (two- to threefold). This stimulation of IE-NCAT expression is not observed upon coexpression of pPstI-N (Bgl), which lacks IE-N activity. The autoregulation of IE-NCAT in the presence of intact IE-N, therefore, appears to be the effect of the IE-N gene product and not the effect of competition for *trans*-acting regulatory factors by the two promoter elements.

The results presented here indicate that transient expression of IE-N is regulated by at least three viral factors in addition to host factors. Further experimentation will be required to determine whether expression of IE-N in infected cells is mediated by the same viral factors.

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